Estimation of Aquaporin-4 levels in cerebral cortex and its role in brain oedema and neurological function in an automated cortical cryoinjury model in mice

Dissertation submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, for the M.Ch. Neurosurgery part II Examination, August 2014

CERTIFICATE

This is to certify that the dissertation entitled **— Estimation of Aquaporin-4 levels in cerebral cortex and its role in brain oedema and neurological function in an automated cortical cryoinjury model in mice** is the bonafide original work of **Dr. G. Edmond Jonathan**, Christian Medical College, Vellore submitted in partial fulfillment of the rules and regulations, for Branch-II M.Ch. Neurosurgery, Part-II examination of the Tamil Nadu Dr. M.G.R. Medical University to be held in August 2014 under my guidance and supervision during the academic year 2009-2014

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AIMS AND OBJECTIVES

1) To study the spatial and temporal profile of expression of aquaporin-4 (AQP-4) at the injury site and distant sites from the injury site at 24, 48, 72 hours post injury following cerebral cortical cryoinjury in mice.

2) To correlate the AQP-4 levels at site of injury with the neurological function at various different time intervals following injury.

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June 25, 2012

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Sub: **Fluid Research grant project NEW PROPOSAL :(Animal Study)**
Estimation of Aquaporin-4 levels in cerebral cortex in a murine model of vasogenic cerebral edema
Dr. G. Edmond Jonathan, Post Graduate Registrar, Neurological Sciences, Dr. V Rajashekhar, Dr. Ranjith K. Moorthy, Dr. Anna Oommen, Neurological Sciences, Ms. Christina Isaac, Ms. Thressi Maxwell, Mr. Muthu Kumar, Neurological Sciences.

Ref: IRB Min. No. 7809 dated 18.04.2012

Dear Dr. Jonathan,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Estimation of Aquaporin-4 levels in cerebral cortex in a murine model of vasogenic cerebral edema" on April 18, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Cvs of Drs. Ranjith K Moorthy and V Rajshekar
3. A CD containing documents 1 – 2

We approve the project to be conducted as presented.

This proposal will also need to be submitted to the Institutional Animal Ethics Committee (IAEC) for approval.

INSTITUTIONAL REVIEW BOARD (IRB)
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The animal requirements and budget will have to be discussed with the Animal House Staff prior to submission of the proposal to the Institutional Animal Experimentation Committee.

The Institutional Review Board expects to be informed about the progress of the projects and asks to be provided a copy of the final report.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Secretary
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Mr. Antony Raj, Mr. Ajay Gift Sam and the Central Animal Facility of our hospital to be gracious to help me perform the experiment even during the odd hours.

Mrs. Grace Rebekah from the Department of Biostatistics and Clinical Epidemiology unit who helped in the statistical analysis

The department of Neurological Sciences office staff who have been helpful in providing assistance for resources needed throughout the duration of this project.
I thank the Animal Ethics committee and Institutional Review Board (IRB) of Christian Medical College, Vellore for approving the experiment and the FLUID Research grant for funding this work.

All thanks to God almighty without whom all this would have not been a possibility. I thank my parents, my wife, and my brother last but not least my daughter who have been a constant support through all my proceedings. My special thanks to all my seniors and juniors who have supported me in this project all this far.

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2) To correlate the AQP-4 levels at site of injury with the neurological function at various different time intervals following injury.

INTRODUCTION

In brain, water is continuously shunted between blood-brain and cerebrospinal fluid (CSF)-brain interface. Dysfunction in water homeostasis has deleterious effects on brain function. Cerebral oedema is an inevitable consequence of any significant brain injury and is a common cause for morbidity and mortality in neurosurgical practice (1). Cerebral oedema following traumatic brain injury causes raised intracranial pressure, secondary neuronal damage, brain herniation and death. Osmotic agents, diuretics and steroids are being used in patients to control cerebral oedema following brain surgery, tumors, trauma and stroke (27). Despite this wide variety of agents available, cerebral oedema cannot be treated successfully in a proportion of patients and several mechanisms of oedema formation have not been optimally addressed (28)

REVIEW OF LITERATURE

Cortical cryoinjury model

Klatzo et al (5) have classified cerebral oedema into cytotoxic and vasogenic oedema. In cytotoxic or cellular oedema, the blood brain barrier is intact and there is influx of ions into the astrocytes and neurons with resultant cellular swelling and accumulation of fluid in grey matter. In vasogenic oedema which occurs due to the damage to the blood brain barrier, there is leakage of plasma into the interstitium and there is accumulation of fluid in the white matter. There is less resistance to the flow of the fluid in the white matter compared to the grey matter, as there is parallel alignment of white matter tracts which facilitates easy diffusion of water molecules (12). Vasogenic oedema is the principal type of oedema seen in tumors, trauma, infections and ischemia (2).

Cold injury model by freeze injury has been standardized as a model for vasogenic oedema (2, 29, and 30). Cold injury damages the blood brain barrier causing extravasation of fluid in to the extracellular space.

Vasogenic oedema involves breakdown of the blood brain barrier and extravasation of the plasma into the brain (3). This leads to increased brain volume, raised intracranial pressure, increased extracellular volume and brain displacement.

Vasogenic oedema induced by cold injury is thought to be due to increased solute and water entering the brain following breakdown of blood brain barrier (3). Iso-osmotic fluid and serum proteins enter into the interstitial space from blood stream in response to

hydrostatic pressure gradients (4). In vasogenic oedema there is expansion of the interstitial space (9).

Oedema fluid is cleared by the migration of extracellular water into cerebrospinal fluid (CSF) by the bulk flow in the presence of pressure gradients, glial uptake of protein components of oedema fluid and across ependyma into the ventricles (5). There is reconstruction of the microvasculature architecture and endothelial repair after the cold injury. Repair of the microvasculature is initiated on the third day after cold injury and is entirely regenerated by seventh day (2).

Oedema is maximal on the first day after injury and subsides gradually over the next few days. On the basis of this rationale, in our study we sacrificed the mice at 24 hours when brain oedema is expected to be at its peak and then at 48 and 72 hrs, to study the temporal and spatial profile of AQP-4 expression and its relation to brain oedema and neurological function.

We opted to use the controlled cryoinjury model for the ease of a graded, reproducible range of injury through an alteration of the mechanical force (33).

Pathogenesis of vasogenic oedema

Various pathways have been described to study the pathogenesis of vasogenic oedema in which, the blood brain barrier plays a key role. Its integrity is formed by the endothelial tight junctions.

Various tight junction proteins have recently been identified, such as occludin, claudin, Zona occludin (ZO-1, ZO-2 and ZO-3). Jadhav et al (6) have shown the loss of ZO-1 (the tight junction protein important for maintaining the blood brain barrier)

immunoreactivity in the cerebral microvasculature of the affected tissue in their model of surgically induced brain injury in rats.

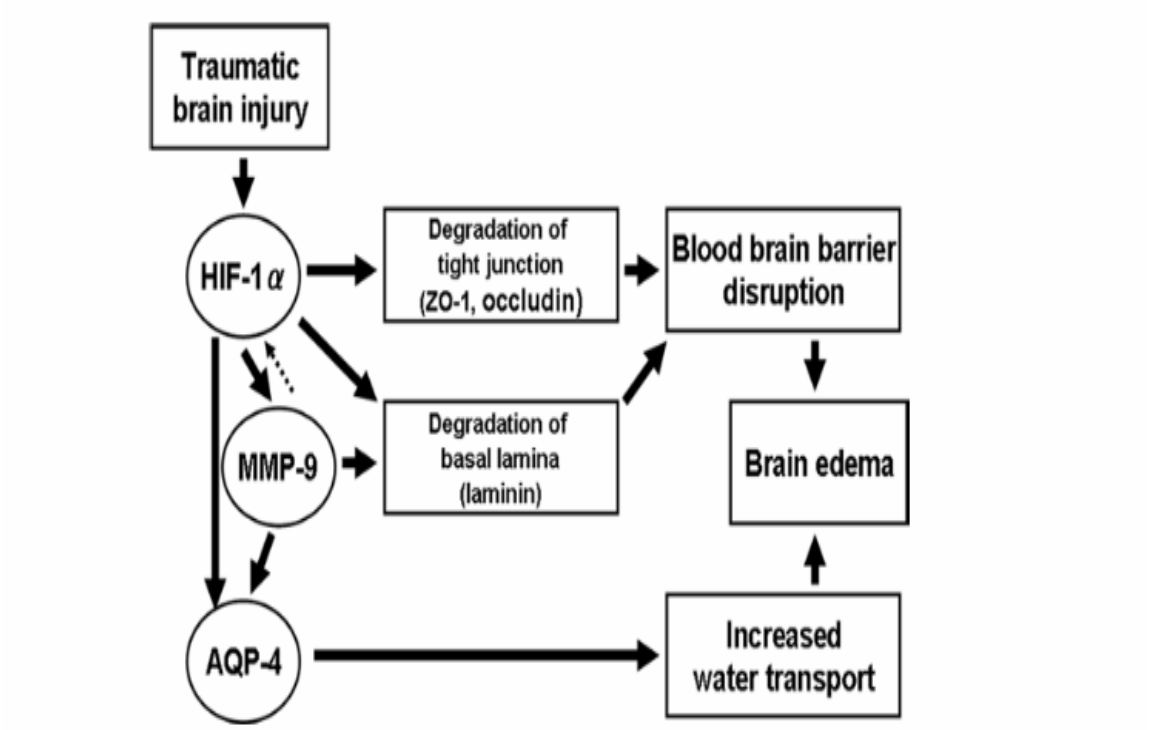
Brain extracellular matrix (ECM) plays a complex role in central nervous system maintenance and function. The two major systems that modify the extracellular matrix (ECM) in the CNS are matrix metalloproteinases (MMPs) and plasminogen activators (PAs) (35). MMPs are a family of zinc endopeptidases which are responsible for the degradation of ECM. They are associated with blood–brain barrier disruption after brain injury, oedema formation and demyelinating process proving detrimental to the CNS. They increase capillary permeability and produce brain oedema that is secondary to ischemic and hemorrhagic brain injury.

The mature CNS normally contains non-detectable or low levels of most MMPs, but they are upregulated in neurological diseases such as multiple sclerosis, malignant glioma, and stroke. In multiple sclerosis, matrix metalloproteinases are thought to play an important role in disease progression owing to their ability to disrupt the blood-brain barrier (BBB) (36). They promote demyelination by direct degradation of the myelin sheath by cleavage of the myelin basic protein (MBP).

MMPs also favour the migration of inflammatory cells into the neuropil, and trigger the release of proinflammatory cytokines IL-6 and TNF. Laminin, a major structural protein essential for maintaining the integrity of the BBB, is cleaved by the matrix metalloproteinases and is responsible for a leaky BBB in the traumatic brain injury (TBI) (28).

Fig. 1 Schematic representation of the pathogenesis of vasogenic oedema in Traumatic brain injury (TBI).

(Adapted from reference28)



Highasida et al (28) concluded in their study that there is increased expression of HIF-1α (hypoxic ischemic factor) in TBI which up-regulates the expression of MMP-9 and aquaporin-4 (AQP-4). MMP-9 acts on the blood brain barrier and causes cerebral oedema. Similarly AQP-4 increases the water transport across the blood brain barrier and plays a role in vasogenic oedema. Saadoun et al (7) found that AQP-4 expression is increased in

oedematous human astrocytomas and that there is significant correlation between the AQP-4 expression and the breakdown of the blood brain barrier.

AQP-4 deficiency ameliorated cytotoxic oedema, whereas there was worsening of vasogenic oedema (38). Papadopoulos et al (37) proposed that VEGF produced by the tumor cells is responsible for causing the brain oedema by opening the tight junctions in the blood brain barrier. Corticosteroids decrease the vasogenic edema by decreaseing the response of vasculature to the VEGF (34).

Src tyrosine kinase phosphorylates and regulates a number of molecules including MMPs, VEGF and plays an important role in brain oedema (39). Src tyrosine kinase regulates VEGF-mediated vascular permeability in the brain following a vascular insult. Suppression of Src tyrosine kinase activity decreases vascular permeability thereby minimizing brain oedema (14, 15).

Paul et al (39) have concluded that there was a decrease in the brain oedema, volume of the lesion and clinical injury with inhibition of src tyrosine kinase in their cryoinjury model.

Newer molecular mechanisms responsible for the oedema are under research which will facilitate the molecule based targeted therapy.

Aquaporin-4 and its role in neuroprotection

Aquaporins

Aquaporin (AQP) belongs to family of transmembrane water channels with selective pores through which water transport occurs in plasma membranes of humans and higher mammals. The first AQP was identified in red blood cells and was called AQP 1(9) and since then 12 AQPs have been described so far in the literature. Agre et al (9) have reviewed the role of AQPs in renal water transport and absorption, CSF formation and reabsorption,

generation of pulmonary secretions, aqueous humor secretion, reabsorption and lacrimation. (9)

AQPs occur as tetramers. Each monomer has its own water pore. The monomer consists of six membrane spanning tilted alpha helical domains with cytoplasmically oriented amino acid and carboxy terminal (10). Most AQPs have 300 amino acids. They are classified in to two groups. AQPs 1, 2,4,5,8 which are permeable to water alone. AQPs 3,6,7,9 which are permeable to water and glycerol and are termed as aquaglyceroporins (11).

Aquaporin-4 in the brain and its isoforms

AQP-1 and AQP-4 are expressed in the brain. The AQP-4 gene is located on chromosome 18 in humans. Its expression is cell cycle dependent and is expressed only in G0/G1 phase (12). Neilsen et al (10) demonstrated expression of AQP-1 in the choroid plexus where it plays a role in CSF production. Papadopoulos et al (12) described AQP-4 as the main water channel in the brain. It is expressed in the astrocytic foot processes surrounding the capillaries and the glial limiting membrane of the brain. It is also found in the astrocytes and ependymal cells throughout the brain and spinal cord at the pial and the ependymal surfaces which are in contact with CSF in subarachnoid spaces and ventricles (13).

Neilsen et al (10) concluded that perivascular glial processes and glia limitans are the primary sites of water flux due to high concentration of AQP-4. The pattern of expression of aquaporin is same in the human and the mice brain. Neonates have less prominent AQP-4 expression in the brain (4).

AQP-4 has different isoforms based on the length of NH2 terminal. The shorter form is AQP-4 M23. It forms regular large membrane aggregates known as Orthogonal Array of Proteins (OAP) which are seen as regularly spread intramembranous particles in free fracture

electron microscopy. It is the most abundant isoform of AQP-4 in the brain. It has the highest water permeability compared to AQP-4 M1.The longer isoform is labelled as AQP-4 M1 (14).

Figure 2 Structure of AQP-4 with the six Alpha helices (labelled I to VI). Note the carboxy (C) and amino (N) terminal

(Adapted from reference 51)

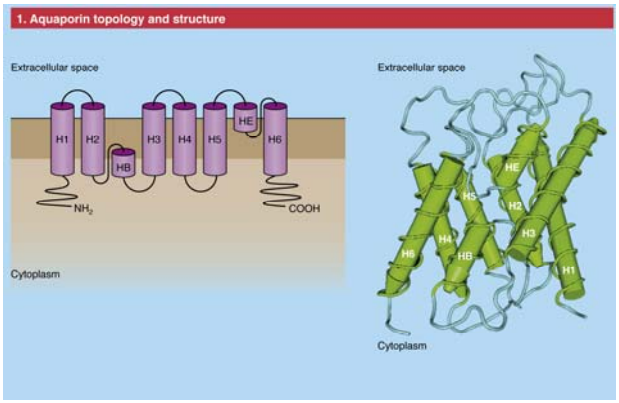
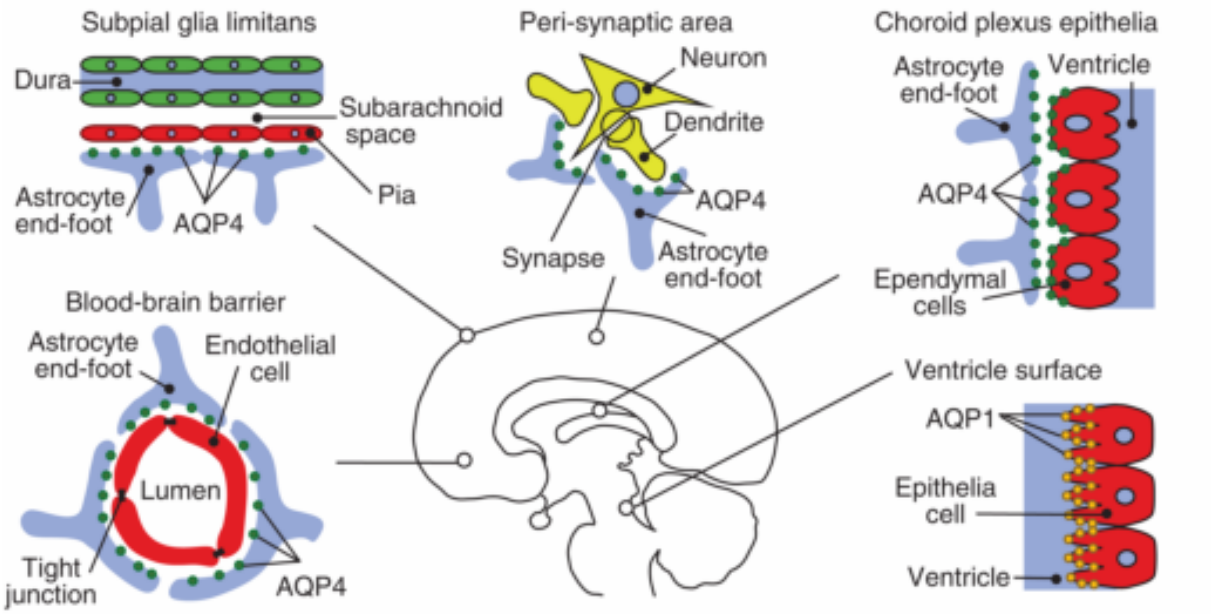


Figure 3 Distribution of AQP-4 in the brain

(Adapted from reference 48)



Detection of AQP-4

AQP-4 in the brain has been detected in vitro using western blotting technique, ELISA, immunofluorescence staining and immunohistochemistry.

The sensitivity and specificity of western blot for detecting the antibodies against AQP-4 is 81% and 97 % (49). The sensitivity and specificity of immunofluorescence staining is 91% and100%. The sensitivity and specificity of immunohistochemistry to detect AQP-4 antibodies is 86% and 100 % (50). We preferred to use western blotting as it is easily reproducible and protein specific and provide an opportunity for quantitative estimation.

Role of Aquaporin-4

AQP-4 water channel is bidirectional. Astrocytes regulate water balance in the normal brain by altering the levels of AQP4 expression. Papadopoulos et al (12) have proposed that in pathological conditions of brain oedema, increased AQP-4 can contribute to the formation and also to the resolution of oedema. It allows the clearance of excess fluid in the vasogenic oedema and causes the accumulation of fluid in cytotoxic oedema (13). There is up-regulation of AQP-4 in brain tumors, stroke, and traumatic brain injury (12, 16).

There is up-regulation of mRNA of AQP-4 following disruption of the blood brain barrier (12, 16). Experiments on the AQP-4 null mice demonstrated the role of AQP-4 in cerebral oedema. AQP-4 null mice are grossly phenotypically normal and do not have neurological abnormalities. They have normal blood brain barrier and osmoregulation (13).

Verkman et al (13) showed that AQP-4 null mice did not show any impairment in the brain development and blood brain barrier integrity. Data from immunostaining and protein quantification in human and animal studies showed up-regulation of AQP-4 in brain tumors, stroke and after traumatic brain injury (7, 15, 16, 17, 20, 23, 24, 25, 26)

Aquaporin-4 and Vasogenic oedema

Verkmann and Papadopoulos (12) reported worse clinical and pathological outcome with higher intracranial pressure in AQP-4 deficient mice subjected to cryoinjury (20). AQP-4 null mice had more brain swelling compared with wild type after cortical freeze injury and brain tumor implantation (20). Deletion of AQP-4 in vasogenic oedema reduces the rate of water flow from the brain. AQP-4 levels are increased in traumatic brain injury.

Sun et al (15) reported increased expression of AQP-4 at the site of the injury in rats following cortical impact injury. There was exponential increase in the level of AQP-4 with time that peaked 24 hours post injury. There was decrease in the expression of AQP-4 in the cortex adjacent to the injury. Similar findings have been reported by others (15).

Hu et al (16) in their study in the expression of AQP-4 in traumatic brain injury in human brain specimens found that there was no increase in the AQP4 levels 6-14 hours after the injury at the site of the lesion and this peaked at 24 hours post injury. AQP-4 immunoexpression was demonstrable up to 8 days post injury. There was no AQP-4 immunoreactivity adjacent to the lesion. There was significant increase in the levels of AQP-4, 15 hours after the injury and peaked at 24 hrs after the injury at the injury site (16)

Neal et al (17) using a penetrating brain injury in a rat model demonstrated dynamic spatial and temporal changes in AQP-4 expression that contribute to the traumatic brain injury. In their model there was no AQP-4 immunoreactivity in the core of the injury. The perilesional area AQP-4 immunoreactivity was elevated substantially at 24hrs, 72 hrs after the injury. There was increase in the AQP-4 immunoreactivity in the contralateral hemisphere 24 hours after the injury (17). Manley et al (18) have demonstrated increased AQP-4 levels in the ischemic penumbra helps in eliminating the extracellular water.

Progesterone administration following traumatic brain injury in rats decreased brain tissue water content at 24 hrs and 72 hrs after the injury by stabilizing the AQP-4 expression in the injured areas. It also resulted in increased AQP-4 expression in the hypothalamus and regions surrounding the third ventricle mediating maintenance of osmotic equilibrium (19).

In hydrocephalus models, AQP-4 null mice had accelerated progression of ventricular enlargement compared to the wild type mice due to the reduced water permeability of the ependymal layer, sub ependymal astrocytes, and glia limitans that resulted in reduced elimination rate of CSF across these borders in to the subarachnoid space (4).

Increased AQP-4 expression has also been correlated with increased severity of the hydrocephalus in a rat model of communicating hydrocephalus (21). AQP-4 expression is upregulated in astrocytes around oedematous brain tumors (7, 20) in brain contusions, bacterial meningitis and infarcted brain and in brain injury and after mannitol administration. AQP-4 has also been shown to have a beneficial effect in reduction of oedema following Staphylococcal brain abscess.

To summarize, many animal studies have proved that AQP-4 was beneficial in vasogenic brain oedema; and AQP-4 expression studies in humans revealed that AQP-4 was responsible for perilesional edema and contrast enhancement in gliomas and meningiomas while also being responsible for oedema resolution following traumatic brain injury.

The role of AQP-4 in experimental models as well as human brain tumours has been summarized in Table 1 and Table 2.

Selective enhancement and suppression of AQP-4 activity in particular disease states aids in formation as well as clearance of oedema from any cause. Thus providing an effective

therapeutic option to intervene at any stage of manifestation of the disease. Altering AQP-4 expression could serve as a putative target for reducing cerebral oedema refractory to all other agents.

AQP-4 is an important protein that plays a major role in maintaining the water homeostasis in the brain and a review of literature suggests that it has a role in early resolution of vasogenic oedema. Although the spatial and temporal profile of AQP-4 following injury has been measured, its role in preserving neurological function has not been correlated with this spatio-temporal profile. We propose to validate the spatio temporal profile of AQP-4 following cerebral cortical cryoinjury and study its relation to neurological function in the short term following cryoinjury.

Table 1 Role of AQP-4 in animal models of trauma

Study	**Animals**	**Model of Injury**	**AQP-4**	**Time**	**Oedema**	**Effect**
Ding et al(22)	Rats	TBI (weight drop)-MW, 450gm, 2m	Increased (HIF-1alpha)	1,4,24,48hrs	Not studied	
Sun et al(15)	Rats	TBI 800gm/cm, 3mm	Increased in center and decreased in the adjacent areas	1-4-24 hrs	Increased at injury site (wet vs dry brain)	Up regulation of AQP-4 in TBI
Papadopolus et al(20)	Mice AQP-4KnKM	Freeze injury, Melanoma model			Increased	Higher ICP,Oedema, worse clinical outcome using mouse clinical scales
Zhao et al(23)	Rats	TBI (CCI) 6m/s, 2mm	Increased in periphery	1,3 days	Increased (wet vs dry method)	Increased AQP-4 clears oedema
Neal et al(17)	Rats	PBI	Increased n periinjury site	24,72 hrs	Not studied	Studied spatial and temporal expression

Table 2 Estimation of AQP-4 in human brain samples.

Study	**Humans**	**Increased (AQP4)**	**Odema**	**Conclusion**
Saddoun et al (7)	Glioma	Increased expression in the gliomas and the surrounding brain.	Increased Vasogenic odema, disrupted BBB	Increased AQP-4 with gliomas.
Ng et al (24)	Meningioma	Increased in the tumors and the surrounding brain	MRI brain was used to study oedema	Increased AQP-4 in Oedematous Meningioma
Suzuki et al (25)	Contusion	Increased at injury and surrounding injury	Increased by histopathology (Histology)	Increased AQP-4 helps in oedema clearance
Wang et al (26)	Meningioma	Increased AQP-4 and VEGF in the meningiomas	(MRI-T2w Flair) was used to measure oedema	Increased AQP-4 in Oedematous meningioma.
Hu et al (16)	Contusion and Tumors	Increased at the site of the injury.	Oedema was measured by CT and histopathylogy.	Increased levels with TBI and Tumors.

TBI-Traumatic brain injury, PBI-Peneterating brain injury,CCI-Controlled cortical impact injury,HIF-Hypoxemia ischemic factor, KnKm-Knockout mice,AQP-4 – Aquaporin-4CT-Computed tomography;MRI- Magentic resonance imaging: BBB- Blood brain barrier.VEGF-Vascular endothelial growth factor

MATERIALS AND METHODS

The protocol was evaluated and approved by the institutional Review Board and the Animal Ethics Committee of Christian Medical College, Vellore. Funding for the project was provided by the Fluid Research Grant of the institution.

Animals

Young male adult Swiss albino mice weighing 30 to 35 gms were used. Twelve animals each were sacrificed at time points of 24 hours, 48 hours and 72 hours post injury. Brains from six normal mice were used for determining the water content as well as AQP4 distribution in the normal brain.

Creation of cold injury (n=18)

The detailed protocol for creation of automated cryoinjury has been described elsewhere. (40) Briefly, under general anesthesia (Inj. ketamine 100 mg/kg and Inj. xylazine 10 mg/kg), the mice were immobilized on a stereotactic frame (TMB Systems, Germany) after ensuring that the toe pinch withdrawal reflex was lost. The respiratory rate and colour of the extremities were observed during the procedure. A midline incision was made over the scalp to expose the skull from the coronal suture to the lambdoid suture under magnification using an operating microscope.

Using a high speed (35000 rpm) electric dental drill (2mm drill bit), a 5 mm craniotomy was performed just right of midline and in between the coronal suture and lambdoid suture to expose the dura mater. A 3 mm tip cooled hollow copper cylinder (filled with dry ice- acetone mixture to attain a temperature of -50^0 C to -55^0 C) was placed on the intact dura for three minutes. The cylinder was connected to a force tranducer to measure the amount of force delivered and this was standardized.

A force transducer attached to the copper cylinder, the amount of force on placing the probe on the dura and depressing it by 1mm was standardized to 10gm weight and this was measured each time the injury was performed, as depicted in Figure 4.

After wound closure, the animal was returned to its cage.

Sham Injury (n=18)

An equal number of animals were subjected to sham injury under the same anaesthetic protocol wherein the craniotomy was performed and an empty (non pre-cooled) copper cylinder tip placed on the intact dura.

Fig 4. A B C

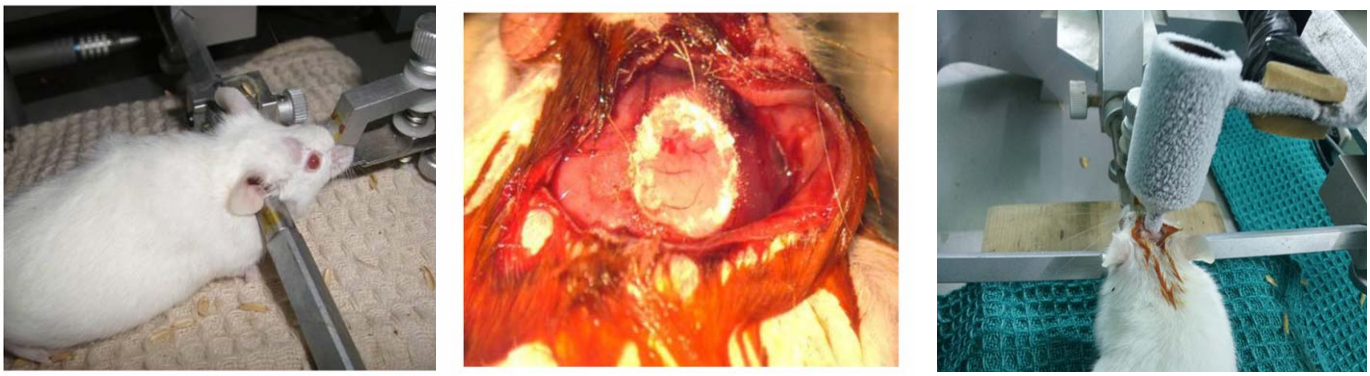


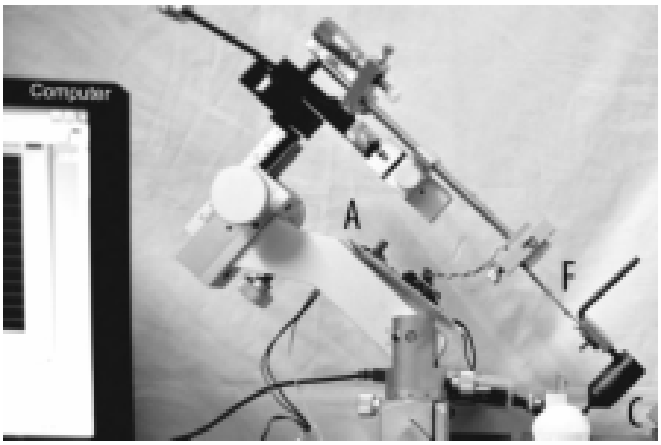
Figure 4 A Mouse fixed to the Stereotactic frame.

Figure 4 B 5 mm craniotomy made between the lambda and bregma. Note that the dura is kept intact.

Figure 4 C Creation of cold injury by a precooled copper cylinder. Note the frost around the cylinder.

Automated injury model

Photographs showing the setup of the automated injury model (40)



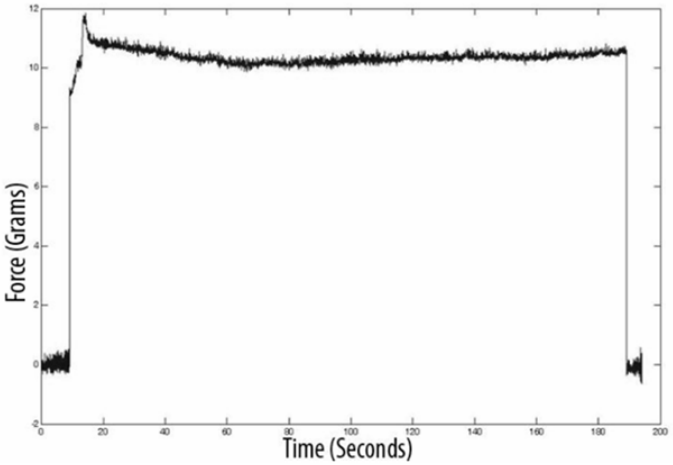


Fig 5 A **Fig 5 B**

Figure 5 A Apparatus with the copper probe (C) connected to the force sensor (F) which transmits the signal to the amplifier (A)

Figure 5 B Graphical representation of the constant force applied for three minutes to create the injury

Figure 6 Schematic representation of the automated cryoinjury model (40).

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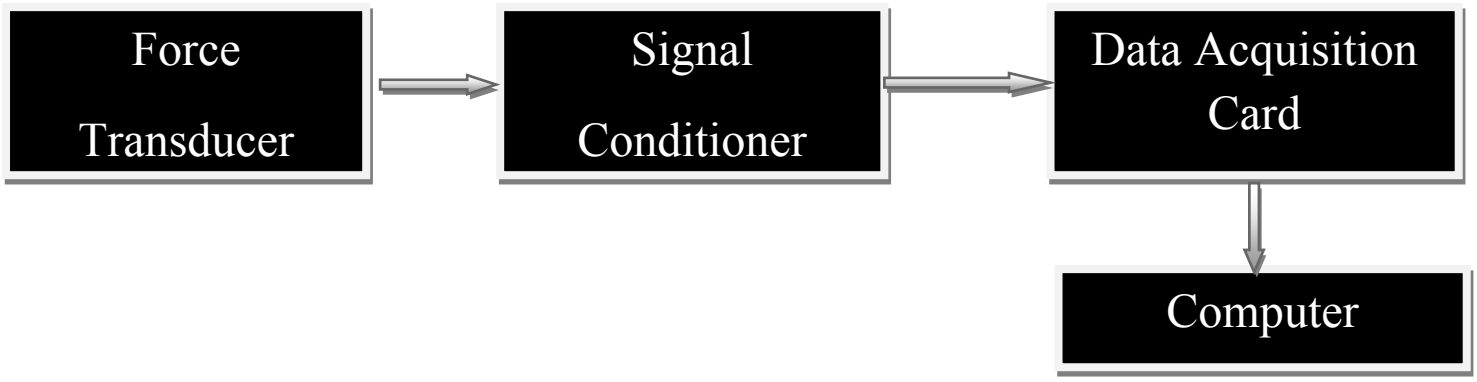


Control Group (n=6)

These mice were not subjected to any anaesthesia or injury. They were sacrificed to determine the expression of AQP-4 in normal brain and to determine the normal dry weight and wet weight of the mice.

Six mice each in the sham as well as experimental groups were sacrificed at 24, 48 and, 72 hours following injury to estimate AQP-4 expression and percentage water content of the brain.

Evaluation of functional outcome

Neurological status of the mice was evaluated using the Neurological Severity scale (NSS) (shown in Figure 6). pre-injury and at 24 hours, 48 and 72 hours post injury by an independent observer blinded to the nature of surgery the animal had. The animal was scored 0 if it performed the test and was scored 1 if it failed the test.

The animals were subjected to a paradigm consisting of uniform acceleration at 4 rounds per min using the rotarod treadmill. They were pretrained for 3 days before the injury to obtain their baseline scores, and serial measurements were performed thereafter at 24, 48, 72 hours after the injury.

The raw scores (in seconds) obtained on the rotarod treadmill were converted to percentage of the baseline scores to analyze the data.

Table 3 Neurological Severity Scale

Task	Description	Points
Exit circle	Ability to exit the circle of 30 cm diameter within 3 min	0/1
Monoparesis/hemiparesis	Paresis of the upper/ lower limb on the contralateral side	0/1
Straight walk	Alertness initiative and motor ability to walk straight	0/1
Startle reflex	Innate reflex the mouse will bounce in response to loud sound	0/1
Seeking behaviour	Physiologic behavior as a sign of interest in surrounding	0/1
Beam balancing	Ability to balance on a beam of 7x7 mm for atleast 10 sec	0/1
Round stick balance	Ability to balance on a round stick of 5mm for atleast 10 sec	0/1
Beam walk 3 cm	Ability to cross a beam of 30 cm length in 3 min	0/1
Beam walk 2 cm	Ability to cross a beam of 30 cm length in 3 min	0/1
Beam walk 1 cm	Ability to cross a beam of 30 cm length in 3 min	0/1
Maximal score		10

Fig 7 Assessment of the Neurological Severity Scale and Rotarod Score

Assessment of brain water content

Three animals from the experimental group and sham group each were sacrificed at 24 hours, 48 hours and 72 hours post injury with an overdose of inhaled chloroform and their brains were removed and weighed immediately to using an electronic balance with a least count of 0.01 mg to determine the wet weight. The brains were then dehydrated by exposing them to a temperature of 105 ^{0} C for 48 hours in a hot air oven. The dehydrated brains were then weighed to determine the dry weight. The water content of the injured brain was calculated as a percentage using the formula

[(wet weight-dry weight)/wet weight] x 100

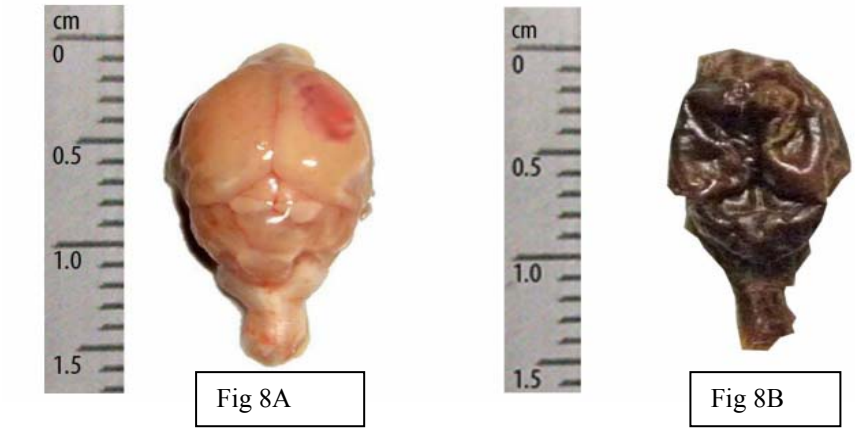


Fig 8A

Fig 8B

Figure 8 A Photograph of the mouse brain immediately after removal from the cranium. Note the presence of a contusion over the right cerebral cortex. The weight of this specimen was measured to be the wet weight**. Fig 8B** Photograph of the mouse brain after 48 hours of incubation at 105degrees. The weight of this specimen was measured to be the dry weight.

Determination of AQP-4 using Western blotting

Western blot analysis was performed as described previously (41). The brain samples were obtained from the site of injury, contralateral hemisphere corresponding to the site of injury and ipsilateral hemisphere adjacent to the injury.

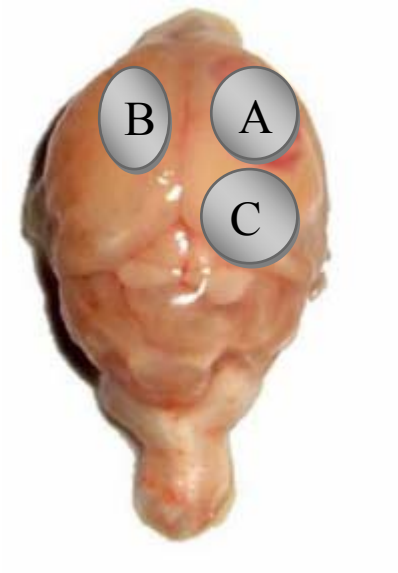


Figure 9 Brain specimen demonstrating the areas chosen for AQP-4 estimation. The actual site of injury is denoted as A, corresponding area in the contralateral hemisphere is denoted as B and area adjacent to the site of injury in ipsilateral hemisphere is denoted C.

Mouse brain tissue from control, sham and injury, were weighed and rapidly frozen by dropping them into liquid nitrogen. Frozen tissues were ground into fine powder using pellet pestle motor with a pre-cooled pestle under liquid nitrogen. To the frozen powder ice-cold Tris buffered saline (TBS) pH-7.4 is added and vortex to get an even suspension. The homogenate was centrifuged for 5 minutes at 1000g at 4^{o}C. Cell pellets were re-suspended in 150µl of NP40 lysis buffer (50mM Tris HCl pH-7.4 containing 250mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4, 1% NP40, 0.02% NaN_3 and 1mM PMSF were added freshly) and incubate for 30mins in ice by vortex for every 10min interval followed by centrifugation for 10min at 13000g at 4^{o}C. Protein concentrations were determined from the supernatant by Lowry's method using bovine serum albumin as the standards.

Equal amount of isolated membrane proteins (20µg) was subjected to reducing 12% SDS-PAGE gel at 100V and electro-transferred to PVDF membrane at 4^{o}C for 1 hour at 80V. Based on the dual color molecular weight standard run, blots were cut into two parts, one part is for AQP4 protein band and the other part is for tubulin protein band (loading control).

Both the blots were blocked with 3% BSA in PBS with 0.1% Tween-20 for 1hr at 25^{o}C and probed with mouse monoclonal AQP-4 primary antibody (1:10000 dilution) to detect AQP4 and mouse monoclonal tubulin primary antibody (1:25000 dilution) to detect tubulin for overnight at 4^{o}C. Further the blots were incubated with goat anti-mouse IgG – Biotin conjugate for 2 hour and streptavidin – HRP for 1 hour at 25^{o}C (at dilution recommended by the manufacturer).

Blots were washed with PBS/T, 3X5 min between steps. Bound HRP was developed with developed with 0.024% H2O2 / 2mM diaminobenzidine in 50mM Tris HCl, pH 7.4 for 10 minutes and the reaction stopped with water. Molecular weight of AQP4 and tubulin bands that developed were determine from blots of dual color molecular weight standard run

and blotted in parallel. Immune bands that develop were imaged and their intensity quantified using QImaging software (version - 7.0.0.5). The arbitrary units obtained were normalized for protein load and expressed as fold change in protein expression with respect to non-injured controls.

All blots included positive and negative controls. The control itself was used as a positive control as AQP-4 is a constitutively expressed protein in the normal brain.

Statistical analysis

Data were expressed as mean ± standard deviation. The percentage water content and Neurological Severity Score (NSS) and RR score was compared between the two study groups using Mann-Whitney U test. P-values of less than 0.05 were considered statistically significant. All statistical analysis was done using SPSS Version 16.0 (IBM, USA).

Results

Percentage water content and its temporal distribution

Variables	**Sham** **Mean±SD**	**Injury** **Mean±SD**	**P values**
Water content (%)			
24Hr	67.80 ± 3.73	79.10 ± 7.57	0.05
48Hr	71.49 ±2.62	77.77 ±6.72	0.13
72Hr	75.26 ± 4.08	81.43 ± 5.67	0.28

Table 4 Illustrates the % water content of brain between the Sham and the Injury groups at 24, 48, 72 hours with respective p values.

The percentage water content of the brain in the injury group at 24 hours post injury was 79.10 ± 7.57 and it was 67.80 ± 3.73 in the sham group, the difference being statistically significant (p=0.05). At 48 and 72 hours post injury, there was no significant difference in the percentage water content between the sham group and injury group. There was a trend of increase in the percentage water content in both the groups with time; however there was significant increase in the water content in the injury group as compared to the sham at the end of 24 hours.

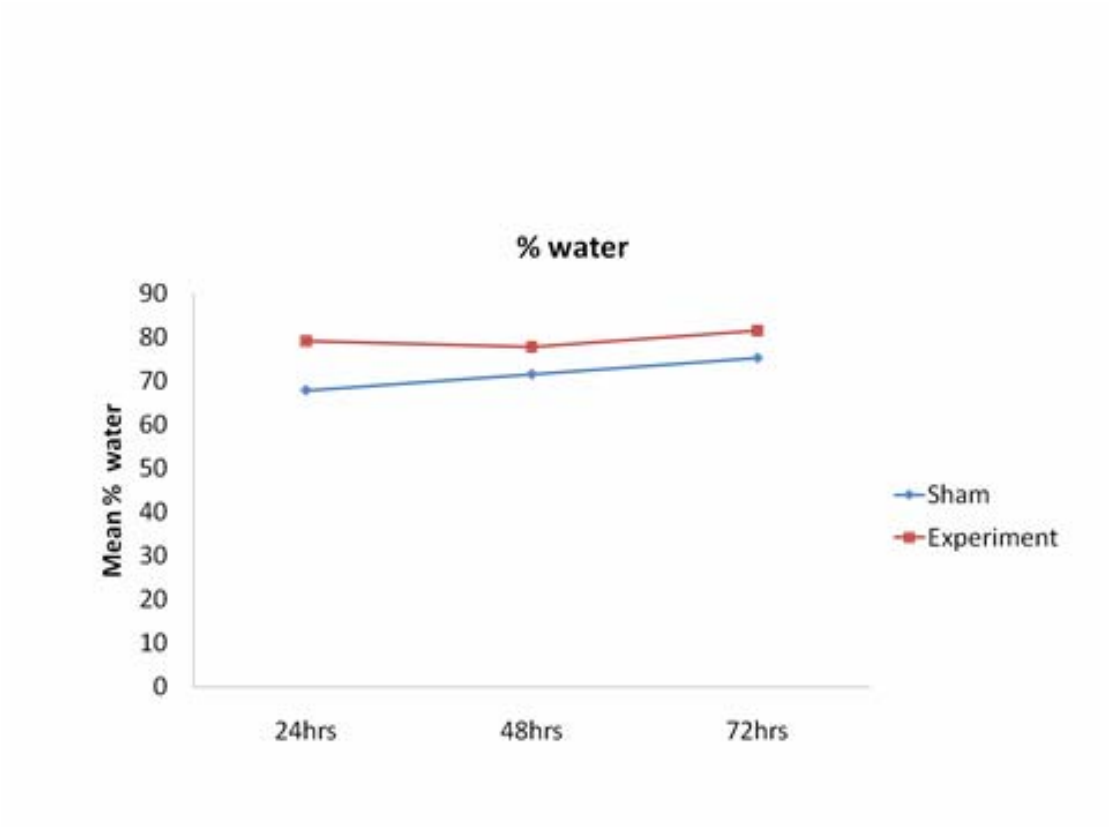


Figure 10 Temporal profile of percentage water content between the sham and injury group

Neurological severity scale (NSS) and Rotarod (RR) score

Variables	**Sham** **Mean±SD**	**Injury** **Mean±SD**	**P values**
Post Injury NSS_24Hr	0.62±0.71	8.50±1.41	0.00
Post Injury NSS_48Hr	0.50±0.67	7.62±1.38	0.00
Post Injury NSS_72Hr	0.38±0.51	6.83±1.47	0.00
Post Injury RR_24Hr	94.02±1.41	46.46±15.38	0.00
Post Injury RR_48Hr	91.04±1.38	53.23±10.39	0.00
Post Injury RR_72Hr	91.53±1.47	61.73±8.02	0.00

Table 5 Neurological severity scale, Rotarod score, between the Sham and the Injury groups at 24, 48, 72 hours and respective p values.

There was significant injury with loss of neurological function in the Injury group when compared to the sham groups. The NSS was 8.50±1.41in the injury group and 0.62±0.71 in the sham group, the difference being statistically significant. (p=0.00) at the end of 24 hours. There was statistically significant difference in the NSS between the injury and the sham group at the end of 48 and 72 hours. The higher NSS was associated with poor neurological outcome. There was gradual improvement in the NSS at the end of 48 and 72 hours in the injury group which was statistically significant.

The RR score was expressed as a percentage; a lower RR was associated with poor neurological outcome. The RR was 46.46±15.38 in the injury group and it was 94.02±1.41 in the sham group. This was statistically significant (p=0.00). Similar results were obtained at 48 and 72 hours. There was trend of improvement in the RR percentage at the end of 48 and 72 hours in the injury group and it almost remained the same in the sham group.

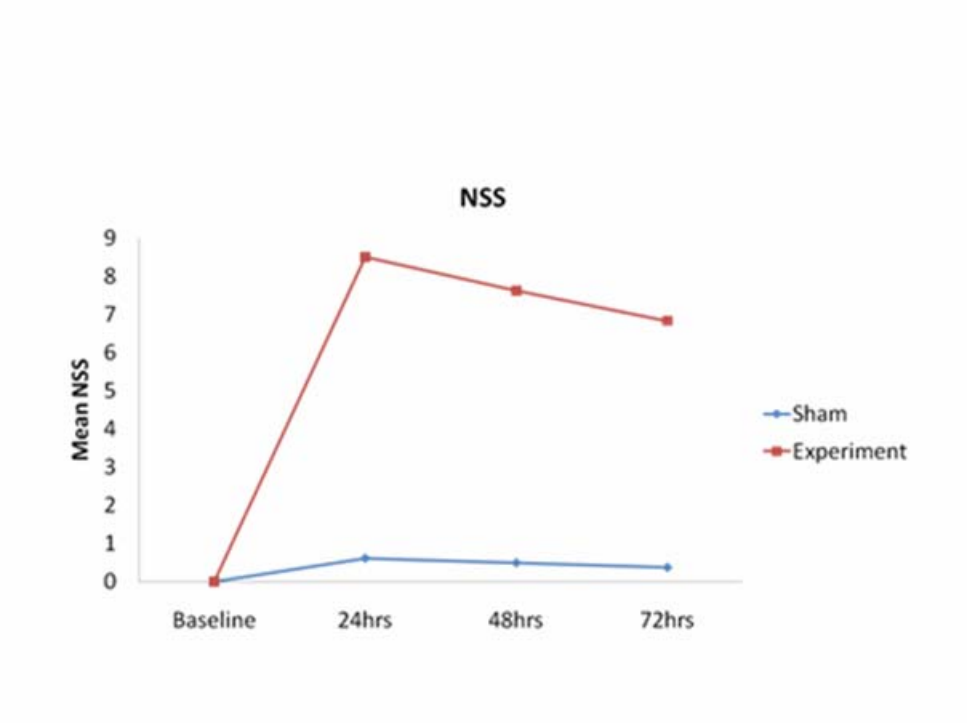


Figure 11 Depicts the NSS at different time intervals between the sham and the injury Group. Note the increased NSS at the end of 24 hours in the injury group and then there is a gradual functional recovery with an improvement in NSS at 48 and 72 hours in the injury group.

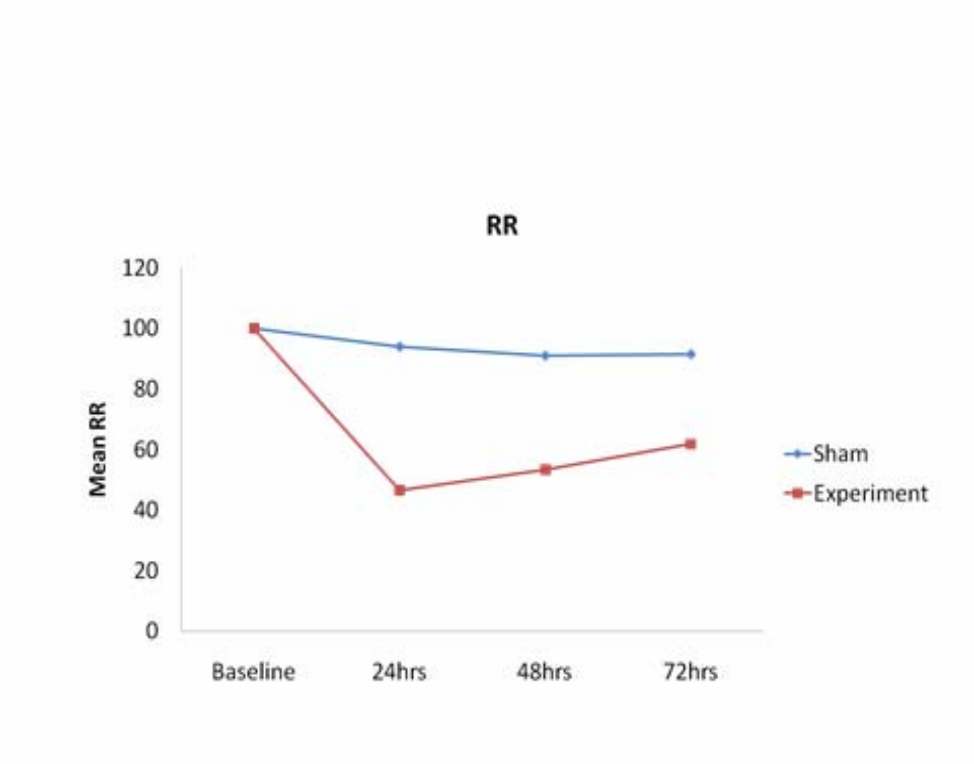


Figure 12 Depicts the Mean RR at different time intervals between the sham and the injury Group. Note the acute fall in the RR percentage at the end of 24 hours in the injury group which correlated to increased percentage water content at the end of 24 hours (Fig 10)

Spatial and temporal expression of AQP-4

Site	Sham Mean±SD	Injury Mean±SD	P value
A			
24 Hours	25.10±1.43	34.39±2.24	0.05
48 Hours	32.81±1.82	29.38±1.61	0.05
72 Hours	25.64±3.59	29.51±2.25	0.13
B			
24 Hours	30.24±1.45	31.46±1.65	0.27
48 Hours	36.05±1.11	24.75±2.68	0.05
72 Hours	24.24±4.17	25.06±1.43	0.83
C			
24 Hours	21.50±2.32	32.77±3.05	0.05
48 Hours	29.55±2.93	27.50±2.77	0.28
72 Hours	27.72±6.00	29.40±1.17	0.83

Table 6 Compares the AQP-4 expression between the sham and the injury group temporal and spatial profile, p values.

Spatial and temporal expression of AQP-4

Site	Control Mean±SD	Injury Mean±SD	P value
A			
24 Hours	26.25±1.14	34.39±2.24	0.05
48 Hours	26.25±1.14	29.38±1.61	0.05
72 Hours	26.25±1.14	29.51±2.25	0.05
B			
24 Hours	21.89±2.53	31.46±1.65	0.05
48 Hours	21.89±2.53	24.75±2.68	0.28
72 Hours	21.89±2.53	25.06±1.43	0.13
C			
24 Hours	22.37±2.78	32.77±3.05	0.05
48 Hours	22.37±2.78	27.50±2.77	0.05
72 Hours	22.37±2.78	29.40±1.17	0.05

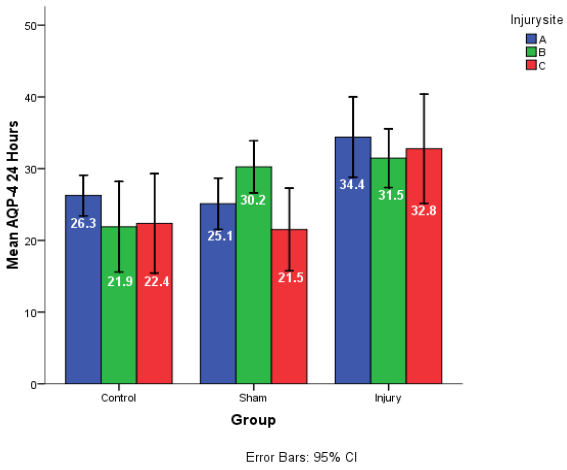
Table 7 Compares the AQP-4 expression, spatial and temporal profile of AQP-4 in the control and the injury group.

Compared to the control group of mice, AQP-4 expression was found to be increased in sham as well as injured animals. There was generalized increased expression of AQP-4 at all three sites (A,B and C) in the injury group at the end of 24 hours, 48 and 72 hrs compared to control group. However, this difference was statistically significant only at 24 hours post injury.

Site	Control Mean±SD	Injury Mean±SD	AQP4 Ratio
A			
24 Hours	26.25±1.14	34.39±2.24	1.34
48 Hours	26.25±1.14	29.38±1.61	1.12
72 Hours	26.25±1.14	29.51±2.25	1.12
B			
24 Hours	21.89±2.53	31.46±1.65	1.43
48 Hours	21.89±2.53	24.75±2.68	1.13
72 Hours	21.89±2.53	25.06±1.43	1.14
C			
24 Hours	22.37±2.78	32.77±3.05	1.46
48 Hours	22.37±2.78	27.50±2.77	1.22
72 Hours	22.37±2.78	29.40±1.17	1.31

Table 8 Compares the increase in the AQP4 in the injury group at different time intervals as compared to controls

The injury mice demonstrated an increase in the number of aquaporin receptors at all time points and at all sites A, B, C when compared to controls, but the highest increase was at 24 hours for all sites of injury. There was a gradual decrease in the aquaporin receptor density over time, but this decrease was less evident at site C. There is at least 1.4 times increase in the AQP-4 fold expression at the injury site and subsequently there is up-regulation of AQP-4 in the opposite hemisphere and the distant sites of injury reaching that of the injury site.



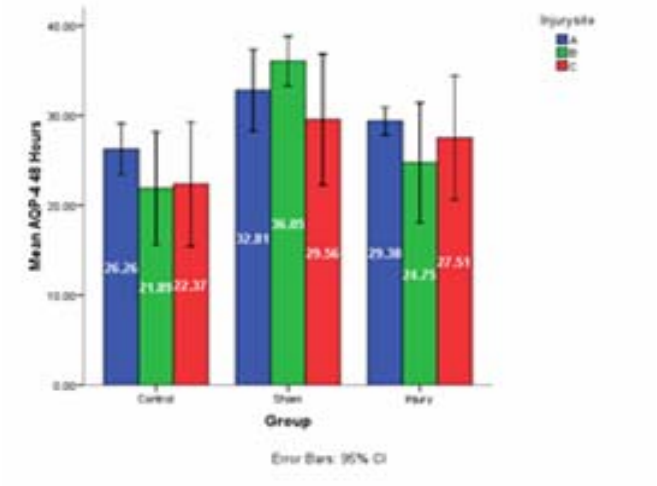
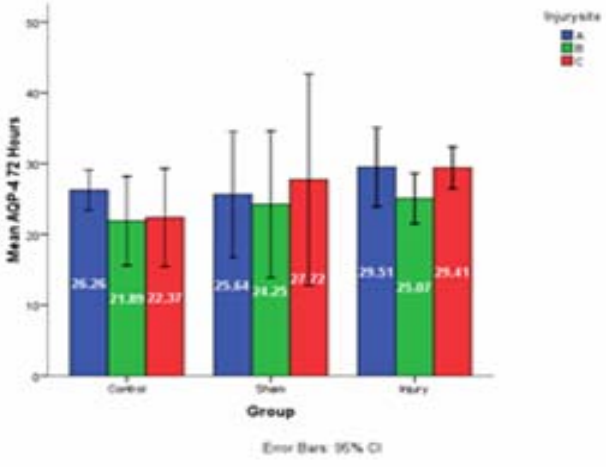


Fig 13 A **B**



C

Figure 13 Graph depicts the spatial profile of AQP-4 expression among normal mice (controls), sham injured and experimental injury groups at 24 hours (A), 48hours (B) and &72 hours (C) post injury.

We are unable to explain the increased expression of AQP-4 in the sham group at the end of 48 hours, the increased expression can be due to the fact that the stress of a craniotomy would have caused the increased expression of AQP-4. The other possible reason could be that anesthesia itself would have resulted in increase in AQP-4. We are conducting experiments to see if the raise in AQP-4 was secondary to administration of anesthesia. There was no cortical injury in this group.

Spatial and temporal expression of AQP-4M1

Site of Injury	Control± SD	Injury ± SD	P value
A			
24 Hours	23.5507 ± 2.56	30.0451± 1.32	0.05
48 Hours	21.2173 ± 2.60	27.4237± 2.72	0.05
72 Hours	16.8840 ± 1.96	26.0814± 2.03	0.05
B			
24 Hours	18.1383± 2.20	28.0001± 2.16	0.05
48 Hours	18.4717± 1.64	26.3568± 1.03	0.05
72 Hours	17.8050± 2.77	25.6306± 1.27	0.05
C			
24 Hours	16.5222± 1.88	29.2949± 1.51	0.05
48 Hours	16.5222± 1.88	25.5266± 0.80	0.05
72 Hours	14.8556± 1.25	25.1941± 1.46	0.05

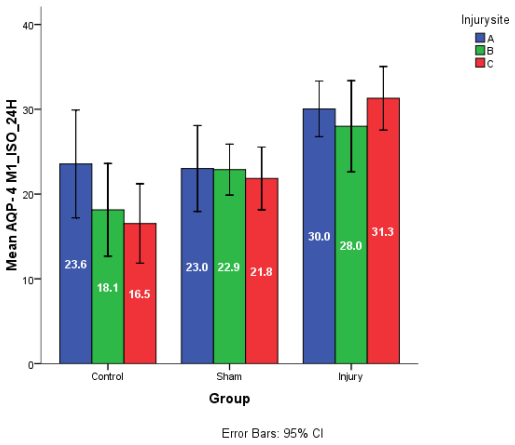
Table 9 Compares the AQP-4 M1 isoform expression between the control and the Injury group temporal and spatial profile, p values. There is increased expression of AQP-4 M1 isoform in the injury group as compared to the control group which was statistically significant at all-time intervals.

Site of Injury	Sham ± SD	Injury ± SD	P value
A			
24 Hours	23.0093± 2.03	30.0451± 1.32	0.05
48 Hours	33.9465± 1.25	27.4237± 2.72	0.05
72 Hours	25.5761± 1.40	26.0814± 2.03	0.51
B			
24 Hours	22.8885± 1.20	28.0001± 2.16	0.05
48 Hours	37.3671± 2.26	26.3568± 1.03	0.06
72 Hours	26.0685± 1.39	25.6306± 1.27	0.51
C			
24 Hours	21.8378± 1.49	31.2949± 1.51	0.05
48 Hours	31.3153± 2.90	25.5266± 0.80	0.05
72 Hours	28.3741± 1.67	27.1941± 1.46	0.27

Table 10 Compares the spatial and temporal profile of AQP-4 M1 isoform in the sham and the injury group. There is generalized increased expression of AQP-4 in all three sites in the injury group at the end of 24 hours, 48 and 72 hrs.

Site of Injury	Control± SD	Injury ± SD	AQP-4M1 Ratio
A			
24 Hours	23.5507 ± 2.56	30.0451± 1.32	1.27
48 Hours	21.2173 ± 2.60	27.4237± 2.72	1.29
72 Hours	16.8840 ± 1.96	26.0814± 2.03	1.54
B			
24 Hours	18.1383± 2.20	28.0001± 2.16	1.54
48 Hours	18.4717± 1.64	26.3568± 1.03	1.42
72 Hours	17.8050± 2.77	25.6306± 1.27	1.43
C			
24 Hours	16.5222± 1.88	29.2949± 1.51	1.77
48 Hours	16.5222± 1.88	25.5266± 0.80	1.54
72 Hours	14.8556± 1.25	25.1941± 1.46	1.69

Table 11 Compares the increase in the AQP-4M1 in the injury group at different time intervals as compared to controls



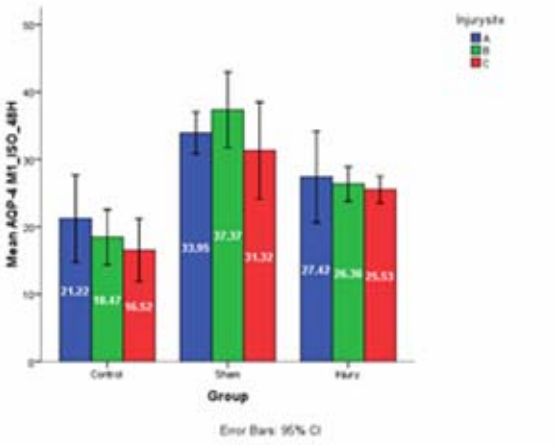
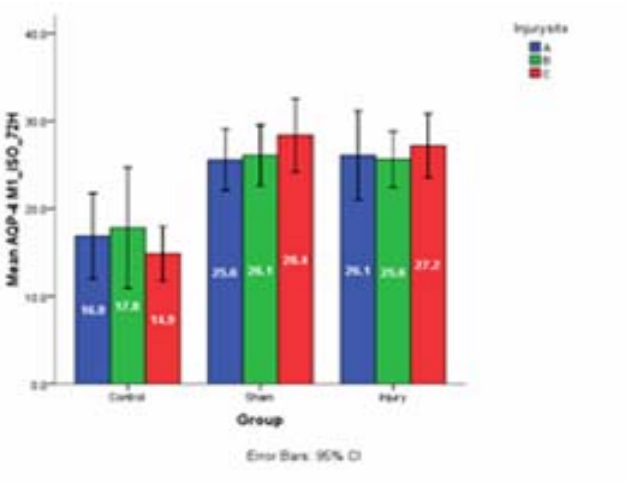


Fig 14 **A** **B**



C

Figure 14 Graph depicts the AQP-4 M1 isoform expression amongst the Control: Sham: Injury Groups at different sites of Injury at 24 Hours(A), 48 hours (B) and 72 hours (C). There is increased expression of the AQP-4 M1 isoform in the injury group at site of injury as compared to the sham and the control at the end of 24 hours which was statistically significant

Spatial and temporal profile of AQP-4 M23

Site of Injury	Control ± SD	Injury ± SD	P value
A			
24 Hours	16.6870± 1.45	28.1773± 1.26	0.05
48 Hours	15.5267± 1.06	27.3410± 2.30	0.05
72 Hours	16.9715± 1.13	28.8212± 1.82	0.05
B			
24 Hours	17.2261± 2.07	25.1432± 1.86	0.05
48 Hours	18.1151± 2.04	25.7855± 2.14	0.05
72 Hours	17.6393± 2.54	27.9759± 1.01	0.05
C			
24 Hours	13.2049± 2.34	26.9030± 3.40	0.05
48 Hours	13.4163± 2.58	25.2912± 1.50	0.05
72 Hours	12.6764± 1.91	27.6217± 1.35	0.05

Table 12 Compares the control and injury group, the AQP-4 M23 expression, temporal and spatial profile, p values. There is increased expression of AQP-4 M 23 in the injury group in all the three sites as compared to the control group at 24,48,72 hours. This was statistically significant at all-time points

Site of Injury	Sham ± SD	Injury ± SD	P value
A			
24 Hours	22.4719 ± 2.09	28.1773+ 1.26	0.05
48 Hours	24.5107 ± 1.18	27.3410+ 2.30	0.27
72 Hours	27.1363 ± 2.15	28.8212+ 1.82	0.27
B			
24 Hours	22.4014 ± 2.23	25.1432+ 1.86	0.12
48 Hours	23.6674 ± 1.16	25.7855+ 2.14	0.27
72 Hours	25.6643 ± 2.13	27.9759+ 1.01	0.12
C			
24 Hours	18.5940 ± 2.22	26.9030+ 3.40	0.05
48 Hours	27.4307± 1.54	25.2912+ 1.50	0.12
72 Hours	24.1371 ± 1.78	27.6217+ 1.35	0.12

Table 13 Compares the sham and injury groups, the AQP-4M23 expression, temporal and spatial profile, p values. There is increased expression of AQP-4 M23 at the site A and C in the injury group which was statistically significant as compared to the sham group at the end of 24 hours.

Site of Injury	Control ± SD	Injury ± SD	AQP-4M23 Ratio
A			
24 Hours	16.6870± 1.45	28.1773± 1.26	1.69
48 Hours	15.5267± 1.06	27.3410± 2.30	1.76
72 Hours	16.9715± 1.13	28.8212± 1.82	1.69
B			
24 Hours	17.2261± 2.07	25.1432± 1.86	1.45
48 Hours	18.1151± 2.04	25.7855± 2.14	1.42
72 Hours	17.6393± 2.54	27.9759± 1.01	1.58
C			
24 Hours	13.2049± 2.34	26.9030± 3.40	2.03
48 Hours	13.4163± 2.58	25.2912± 1.50	1.88
72 Hours	12.6764± 1.91	27.6217± 1.35	2.17

Table 14 Compares the increase in the AQP-4M23 in the injury group at different time intervals as compared to controls

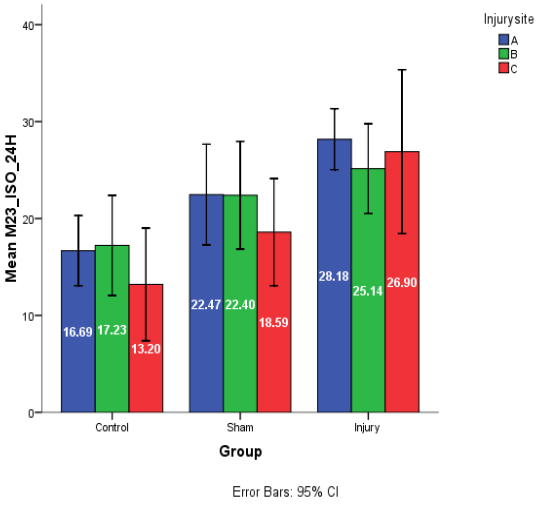
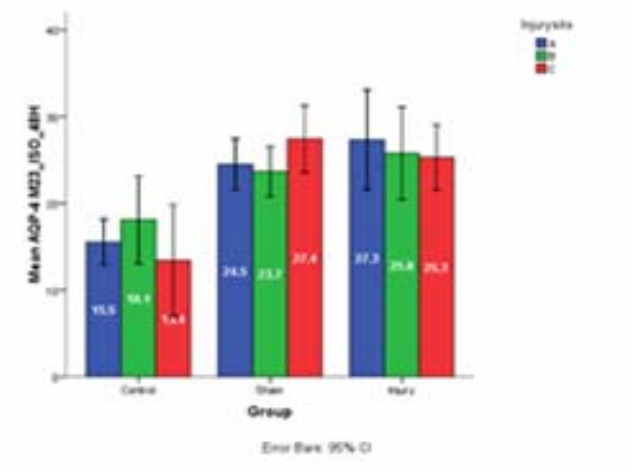
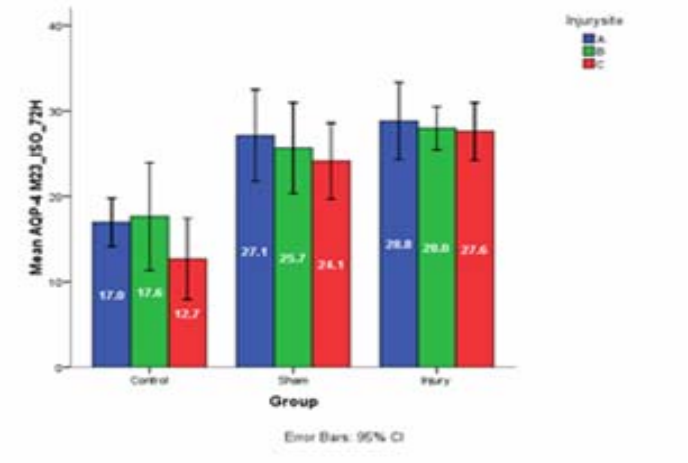


Fig 15 A



B



C

Figure 15 Graph depicts the AQP-4 M23 isoform expression amongst the Control: Sham: Injury Groups at different sites of Injury at 24 hours (A) , 48 hours (B) and 72 hours (C).

Table15 *Comparing the AQP4 levels in the injury group at the site of injury (A), opposite site of injury (B) and peri-injury site (C)*

	A	**B (% of A)**	**C (% of A)**
24hours	34.39	31.46 (91.48)	32.77 (95.28)
48hours	29.38	24.75 (84.24)	27.50 (93.6)
72hours	29.51	25.06 (84.9)	29.40 (99.6)

The mean AQP4 density decreased from site A to C at 24,48 and 72 hrs for all injury points, however the nadir was a point B. At C, the density had recovered to the levels at A at 72hrs

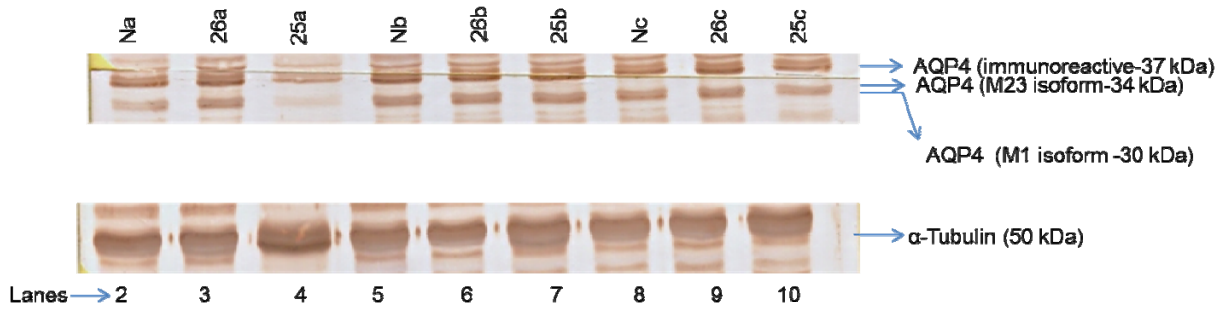
Table 16 *Comparing the AQP4M1 levels in the injury group at the site of injury (A), opposite site of injury (B) and peri-injury site (C)*

	A	**B (% of A)**	**C (% of A)**
24hours	30.04	28.0 (93.3)	29.29 (97.5)
48hours	27.42	26.35 (96.09)	25.52 (93.07)
72hours	26.08	25.63 (98.27)	25.19 (96.58)

Table17 *Comparing the AQP4M23 levels in the injury group at the site of injury (A), opposite site of injury (B) and peri-injury site (C)*

	A	**B (% of A)**	**C (% of A)**
24hours	28.17	25.14 (89.2)	26.90 (95.49)
48hours	27.34	25.78 (94.29)	25.29 (92.5)
72hours	28.82	27.97 (97.05)	27.62 (95.83)

For both these receptors AQP-4 M1 and AQP-4 M23 the nadir was at point B.



Immunoblot at the end of 24 hours

Figure 16

The tubulin protein band serves as the normal loading control. The Immune bands that developed were imaged and their intensity was quantified using QImaging software (version - 7.0.0.5). The arbitrary units obtained were normalized for protein load and expressed as fold change in protein expression with respect to non-injured controls. Note the increased expression of AQP-4 and its isoforms at the site A(injury site) as compared with site B(opposite to the site of injury from the opposite hemisphere) and site C distant from the site of injury.

DISCUSSION

Automated cryoinjury as a model of vasogenic edema

A number of animal models have been devised to study traumatic brain injury. These models help to understand the pathological process of the traumatic brain injury and help in development of novel therapies. The most commonly used models include the weight drop model, fluid percussion model and the controlled cortical impact model, cryoinjury model.

The cryoinjury model utilizes the application of cold rod to the exposed dura in the mice or rats. A mixture of acetone and the dry ice is used to create a cold injury. Pifarre et al (42) and Giralt et al (43) used direct application of dry ice pellet on the dura in their cold injury model. Different grades of injury can be obtained by varying the duration of contact with exposed dura (44).

Cortical cyroinjury causes focal brain injury and break down in the blood brain barrier. Cryogenic brain lesion model is particularly useful for investigating traumatic brain injury-associated blood-brain barrier leakage and vasogenic brain edema. The injury caused by the cryoinjury is focal, well circumscribed and easily reproducible in their size and location (45). This helps in development of the newer molecular targeted therapy and understanding the secondary injury process after the focal injury.

We preferred to use the controlled cryoinjury model as we can induce a graded, reproducible range of injury through an alteration of the mechanical force (33). It also produces a more focal injury than one would expect with a cortical impact / fluid percussion model.

In order to standardize the velocity of impact and the depth of resulting deformation, an automated system was designed by us and was standardized to deliver the cryoinjury which would reduce variations in the depth and deformation of the brain by providing continuous feedback of the force and depth of injury over duration of three minutes. The system designed by us was easily reproducible, cheaper and was adaptable to the stereotactic frame (40).

AQP-4 in injured brain

Many studies have confirmed that AQP4 expression plays an important role in BBB structural changes (10). It is bidirectional. It allows the clearance of excess fluid in the vasogenic oedema and causes the accumulation of fluid in cytotoxic oedema (13). Many studies have demonstrated that up-regulation of AQP4 expression is strongly associated with intracellular oedema (18, 20).

Few studies have shown that up-regulation of AQP-4 was responsible for the clearance of interstitial fluid in the vasogenic oedema which was demonstrated with AQP-4 knockout mice showing worse neurological outcome in the setting of a vasogenic oedema (8, 12, 18). Most researchers believe that the up-regulation of AQP4 expression is the reason for early traumatic brain edema (46, 47).

Temporal distribution of AQP-4

In the present study we found that there was increased percentage water content of the brain, associated with poor neurological outcome in the mice at the end of 24 hours after the injury. This was consistent with results obtained by Papadopoulos et al (20) Lu et al (48) Hu et al (16) and Sun et al (15).

There is up-regulation of mRNA of AQP-4 following disruption of the blood brain barrier (12, 16). Neal et al (17) using their penetrating bran injury model described increased expression of AQP-4 at the periinjury site after 24 hours and 72 hours. They also noticed increased AQP-4 immunoreactivity at the end of 24 hours in the contralateral hemisphere after the penetrating brain inury in the rats (17). Lu et al (48) have observed that the percentage water content and the AQP-4 expression were at its peak at the end of the 24hrs which was similar to our study. However they did not mention about the spatial profile of AQP-4 which was demonstrated by Sun et al (15).

Spatial distribution of AQP-4

Sun et al (15) found increased expression AQP-4 at the site of injury in their animal model, decreased AQP-4 expression adjacent to the site of the injury.

Neal et al (17) studied the temporal and spatial expression of AQP-4 after a TBI in rats and found that there was decreased expression of AQP-4 at the centre of the injury, and an exponential increase in the AQP-4 in the perilesional area at 24, 72 hours.

In the study of Traumatic brain injury by Hu et al(16), they found that there was no increase in the AQP4 levels, 6-14 hours after the injury in the center of the lesion and adjacent to the lesion. But there was significant increase in the levels of AQP-4levels, at the site of injury, 15 hours after the injury and peaked after 24 hrs (16). They did not comment on the functional neurological status in their study.

However, we observed uniform increase in AQP-4 expression at the site of injury as well as distant sites. There was a marked increased expression of AQP-4 at the injury site. This was significant at the end of 24 hours. There was no decrease in the AQP-4 in the adjacent brain as reported by Sun et al (15). Guo et al (19) concluded in their study that

increased expression of AQP-4 at the proximal and distal sites of injury was responsible for the development of cerebral edema following a traumatic brain injury.

Saadoun et al (7) have found that there was increased expression of AQP-4 in edematous brain tumors like high grade astrocytomas and metastatic carcinomas. Similar results were obtained by Hu et al (16) who found increased expression of AQP-4 in edematous brain tumors.

Correlation of AQP-4 expression with brain edema and neurological function

We found in our present study that increased levels of AQP-4 at the end of 24 hours in the injury and the periinjury sites was responsible for the increased brain water content at the end of 24 hours. This was associated with poor neurological function. The fall in AQP-4 levels at the end of 48 and 72 hours correlated with recovery of the neurological function. This was consistent with results published by Papadopoulos et al (20) who noticed that increased expression of AQP-4 after the traumatic brain injury was associated with worse neurological function at the end of 24 hours.

However Zhao et al (23) found that increased expression of AQP-4 was associated with clearance of edema fluid after a traumatic brain injury. Sun et al (16) concluded that increased expression of AQP-4 was responsible for the brain edema after traumatic brain injury.

We found in the present study that there was gradual decline in the levels of AQP-4 in the injured mice at 48 and 72 hrs which was associated with improved neurological status. After 24 and 48 hours the increase in the water content of the brain was not statistically significant as it was at the end of the 24 hours this could be attributed to the gradual fall in the AQP-4 receptors over a period of time and the cause of the edema could be due to the other

factors other than the AQP-4. This study is unique as it compares the spatial and temporal profile of the AQP-4 and its role in cerebral edema and neurological outcome.

CONCLUSION

There is a 1.4 fold increase in AQP-4 expression in the injured brain as compared to sham as well as controls at the first 24 hours following injury that could be correlated with deterioration in functional outcome as well as development of brain oedema. Over the next 48 hours, there was partial functional recovery with reduction in AQP-4 expression. Though there was increase in the percentage water content at the end of 48 and 72 hours there was no statistically significant increase in the water content as that seen at the end of 24 hours. Hence newer strategies to target AQP-4 during the early hours of traumatic brain injury could lead to better treatment of cerebral oedema following a traumatic brain injury. However we need more studies to substantiate our findings.

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APPENDIX A

MICE DATA PROFORMA

Date: **Mice No:** **Dots:** **Colour:**

Weight: **Ketamine:** **Xylazine:**

Test Name	Pre Injury	Post injury
Rotarod		
Exit Circle		
Mono / Hemiparesis		
Straight Walk		
Startle Reflex		
Seeking Behaviour		
Beam Balancing		
Roundstick Balance		
Beam Walking-3cm		
Beam Walking-2cm		
Beam Walking-1cm		
Maximum Score		

Wet Weight: **% Water Content of Brain**

Dry Weight: **W-D/W X 100**

Aqp4: